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Induction of heat-shock proteins by glutamine

The 'feeding effect'

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Subconfluent, log-phase Chinese hamster ovary cells induced the major heat-shock proteins (hsp) when cells were refed, 40 hours after seeding. This method of inducing heat-shock proteins was also obtained by refeeding with fresh serum-free media, but not with media with a long shelf life or with media prepared without glutamine. It was observed that addition of glutamine alone to cultures at 40 hours post-seeding induced heat-shock proteins. Addition of ammonium chloride, however, had no discernible effect on heat-shock protein synthesis. Northern blot analysis indicated that this phenomenon reflected an increase in the levels of message for the constitutive/inducible member of the hsp 70 family, but not the non-constitutive member. To determine the effect of this induction on heat sensitivity, unfed and 'heat-shock-induced' refed cultures were heated at 45°C. No significant difference in cell survival was observed. Therefore glutamine is the necessary ingredient required for the induction of heat-shock proteins and this method of inducing heat-shock proteins does not alter heat sensitivity.

Glutamine; Heat shock; CHO cell; Thermotolerance; Cell culture

1. INTRODUCTION

The term 'heat shock' encompasses a variety of greatly differing stimuli which lead to a homologous response in terms of gene expression and protein synthesis. Heat treatments leading to the response range from physiologic, non-lethal to severe and substantially lethal. A large number of non-heat inducers of the heat-shock response have also been examined. However, in most instances these non-heat inducers also represent stressful conditions leading to (usually) substantial cell killing. At the same time, studies have indicated that heat-shock proteins function in the intercompartmental movement, folding and assembly of other proteins and protein ensembles within the cell [1–3]. Clearly, such associated functions represent molecular processes which occur in the cell in the absence of stress. Thus it is important to define conditions which lead to a 'heat-shock' response in the absence of heat or other severe challenges. We demonstrate here that media changes induce the synthesis of heat-shock proteins in CHO cells and identify glutamine as an essential component in the pathway leading to this effect.

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2. MATERIALS AND METHODS

2.1. *Cells*

Cell line CHO (Chinese hamster ovary) initially obtained from R. Tobey (Los Alamos National Laboratory) was cultured in Ham's F-10 media (Grand Island Biological Co, Grand Is., NY) supplemented with 15% newborn calf serum. Other special media used (e.g. methionine-deficient, glutamine-free) were obtained from the same supplier. Cells were routinely seeded at a density of 1.0×10^6 per 10 cm dish and allowed to grow for 40 h at 37°C. At this time cell density was approximately 50% of a confluent state (i.e. approximately 4×10^6 cells/10 cm dish). Cells entered the stationary phase after an additional 15–20 h (i.e. at approximately 60 h post-seeding). Therefore, in the 40–50 h post-seeding interval (when these experiments were performed) the cells were in a state of unrestrained growth. In these experiments the addition of glutamine or the other changes in media described resulted in no alteration in growth rates.

2.2. *Protein radiolabeling and gel electrophoresis*

[^{35}S]Methionine (>800 Ci/mmol) was obtained from Amersham International Ltd., Amersham, UK, was added to methionine-free media at a final level of 10 μCi/ml for use in pulse-labeling experiments. Following stress, cells were washed twice with medium without serum at 4°C, resuspended in Hanks balanced salt solution without calcium and magnesium and containing 1 mM tosyl-L-arginine methyl ester, 1 mM phenylmethyl sulfonylfluoride (PMSF), 5 mM EDTA (Sigma Chemical Co., St. Louis, MO) and sonicated at 4°C. Protein determinations (for gel loading) were made on lysate using the Bradford method [4] and 4× SDS sample buffer was then added to 3 volumes. A discontinuous SDS-polyacrylamide gel electrophoresis (10%) system was used [5]. Gels were stained, dried and exposed to X-ray film (Kodak XAR-5).

2.3. *Northern blot analysis*

The amount of HSP70 mRNA in CHO cells was determined by using the cDNA clone which was derived from constitutive HSP70 gene of HeLa cells, PH.HSP70 (kindly provided by Dr R. Morimoto).

Total cellular RNA was isolated from exponentially growing CHO cells at various times after the addition of 5 mM glutamine using the guanidium method and centrifugation on a 5.7 M cesium chloride cushion. RNA (20 μg) was denatured with glyoxal and dimethyl sulfoxide, and electrophoresed on a 1% agarose gel, and transferred to zeta-probe blotting membrane (Bio-Rad), as described [6]. The probe was labeled using random primers in the presence of Klenow fragment, dNTP, [^{32}P]dCTP, and the labeled cDNA was incubated overnight with the filter at 65°C. The filters were washed at room temperature for 15 min in each of the following solutions: 2×SSC (1× = 0.15 M NaCl and 15 mM sodium citrate, pH 7.0), 0.5× SSC, and 0.1× SSC, all containing 0.1% SDS (sodium dodecyl sulfate) [6]. The filters were then exposed to X-ray film for 2 days at −70°C.

3. RESULTS AND DISCUSSION

In this study CHO cells were routinely seeded as described in section 2 and allowed to grow for 36 or 40 h at 37°C (as indicated). At this time the cell density was approximately 50% of a confluent state and cells were in a log-phase state of unrestrained growth. The 40-h 'old' medium was then removed and replaced by full fresh medium containing serum. Fig. 1 presents a pulse-label analysis of the protein synthesis patterns of CHO cells at increasing times after a change of medium. An induction of heat-shock proteins of 70 and 90 kDa was observed as a result of this refeeding, becoming evident at approximately 4 h after the change and disappearing at later times. The 70 kDa species appeared to migrate with the constitutive/inducible member of this family (also referred to as hsc 70 or hsp 73). A less distinctive but in most instances visible induction of the 110 kDa heat-shock protein was also observed.

We next attempted to define the factor(s) in the medium responsible for this effect. These studies are summarized in Table I. Initially the same experiment presented in Fig. 1 was replicated by adding F10 medium without serum. In this case an identical result as seen in Fig. 1 was obtained, indicating that factor(s) in the serum-free medium itself were sufficient to produce this effect. It was also noted in these studies that the age of the medium influenced the induction; i.e. the induction of heat-shock proteins required freshly prepared medium (e.g. prepared within two weeks of use), but was not observed in media with a shelf time of six months. Since the half-life of glutamine is 3 weeks at 4°C and 1 week at 36.5°C [7], medium was replaced at 40 h post-seeding (as described above) with fresh F10 medium prepared without glutamine. In this case, heat-shock proteins were not induced. Conversely, when 5 mM glutamine was added to the medium at 36 h post-seeding, this single component was sufficient to induce a heat-shock response. However, when 1 mM glutamine (the concentration present in freshly made F10 medium) was added, no alteration in heat-shock protein synthesis was observed. This indicated that a lower concentration of glutamine was required for the response with medium change compared to addition of glutamine alone. When glutamine was added to old medium

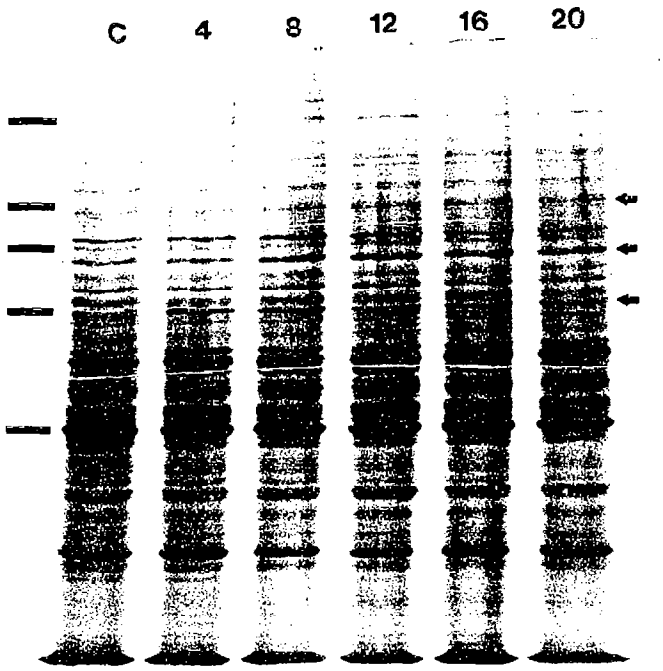

Fig. 1. Polyacrylamide gel electrophoresis of logarythmically growing CHO cells radiolabeled at the indicated hours after a medium change (C, without changing medium). Arrows on the right point to the positions of 110, 89, and 70 kDa heat-shock proteins. The induction of the 70 and 89 kDa proteins are clearly visible. Left margin, molecular weight standards: myosin, 200 kDa; β-galactosidase, 116.5 kDa; phosphorylase B, 92.5 kDa; bovine serum albumin, 66.2 kDa, ovalbumin, 45 kDa.

(which did not itself elicit a response) and the glutamine-fortified old medium added to cells, a heat-shock response was again observed (5 mM was again required). Finally, 12 h after seeding (at which time this 'feeding effect' was not observed) medium was replaced with fresh F-10 medium prepared without glutamine. After 12 h in this medium (total: 24 h post-seeding), glutamine (1 mM) was again added and a heat-shock response was again obtained. That this was due to the 12 h incubation in glutamine-free medium is indicated

Table I

Media constituents*	Induction of HSPs
Fresh F10 with serum	Yes
Fresh F10 without serum	Yes
Fresh F10 without serum & without glutamine	No
Old F10 without serum	No
Old F10 without serum & glutamine added (5 mM)	Yes
Glutamine (5 mM)	Yes
Glutamine (1 mM)	No
Ammonium chloride (5 mM)	No

*Cells were seeded in fresh F10 medium with serum (see section 2) and allowed to grow for 36 or 40 h, at which time cells were refed with the indicated formula.

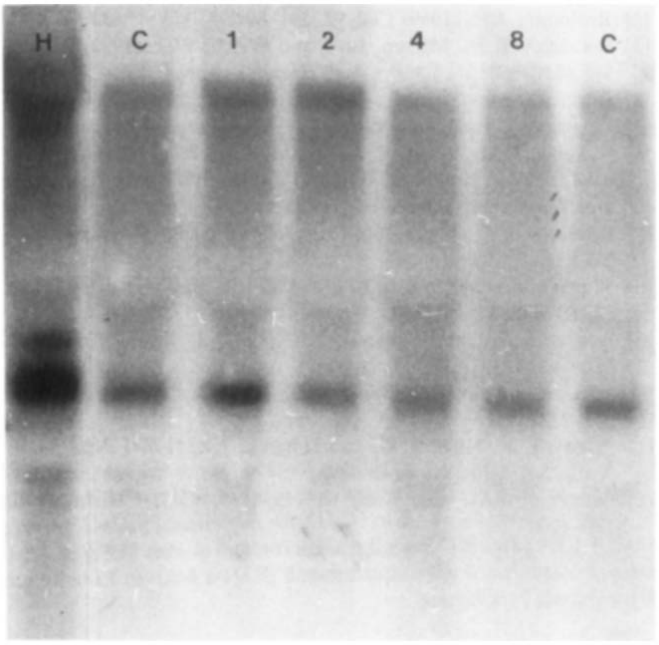

Fig. 2. Northern blot analysis of CHO cells at indicated hours (C, without addition of glutamine) after the addition of 5 mM glutamine using a probe against the constitutive and heat-inducible hsp 70 (also referred to as hsc 70). H, cells heated at 45°C for 10 min and post-incubated for 4 h at 37°C.

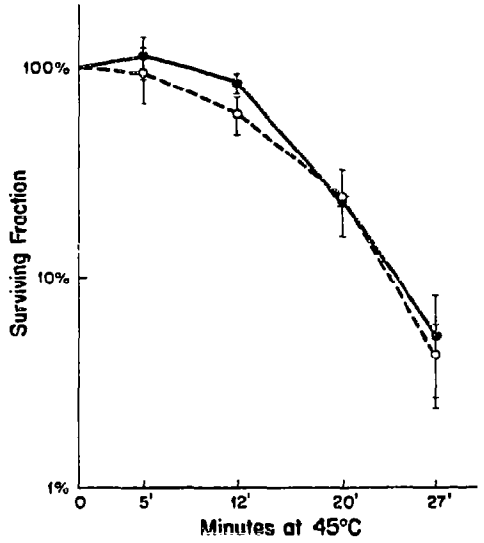

Fig. 3. Survival analysis of heated CHO cells at 8 h after a medium change (○), or without a medium change (●). The induction of heat-shock proteins resulting from refeeding of cells does not significantly alter heat resistance.

by the fact that when cells incubated in full medium were refed at 24 h with full medium, no stress protein induction was evident. These experiments indicate that in the CHO cell line examined here, glutamine is a critical nutrient which controls the expression of heat-shock proteins under non-heat conditions upon medium change. Finally, we considered the possibility that increasing glutamine levels effected cellular ammonia levels, secondarily inducing heat-shock proteins. When 5 mM ammonium chloride was added to cultures, no discernible change in heat-shock protein synthesis was seen.

To examine this result further we determined the effect of glutamine on levels of hsp 70 message. The response of CHO cells to the addition of glutamine at 36 h post-seeding is presented in Fig. 2. An accumulation of message encoding the constitutive hsp 70 member can be seen following the addition of glutamine to the medium. This probe is also seen to weakly hybridize with the less rapidly migrating, homologous message encoding the non-constitutive, inducible form of hsp 70. This message is not increased in control cells and is not induced by the addition of glutamine.

Finally, the induction of heat-shock proteins by heat and other stressful and potentially lethal conditions has been shown to be associated with the induction of the thermal protective phenomenon of thermotolerance. In Fig. 3, a survival analysis of control cells and medium/hsp induced cells, exposed to a 45°C heat challenge delivered at 48 h after seeding or at 8 h after the medium change (performed 40 h after seeding), respectively, is presented. This procedure for the induction of heat-shock proteins does not induce thermotolerance.

In addition to glutamine, other factors may also exist, the deprivation and restoration of which might also lead to an induction of heat-shock protein synthesis and message accumulation. This study defines glutamine as both necessary and sufficient for the 'feeding effect' obtained in routine cell culture work. However, when added in conjunction with fresh media, 1 mM glutamine was sufficient, while 5 mM was required to elicit a response when glutamine was added alone. This indicates that the concentration required is reduced in the presence of fresh medium. Others have described the expression of inducible hsp70 mRNA by the addition of complete serum to serum-starved cells and report that this effect is largely blocked by AraC, an inhibitor of DNA synthesis [8]. In the present study, the addition of glutamine leads to a heat-shock response in an actively proliferating and subconfluent population of cells and would not be expected to be associated with a resumption of DNA synthesis. Earlier studies have demonstrated that restoration of glucose to glucose-starved cells also elicits a heat-shock response [9,10], as does the addition of oxygen to anoxic cells [11]. We have not determined glutamine levels in the experiments where the full medium is changed at 40 h, although adding glutamine to cells incubated for 12 h with glutamine free-media elicited an identical response.

Sanders and Kon have recently demonstrated that in *Drosophila* the induction of heat-shock proteins following heating is enormously enhanced by the presence of

glutamine [12]. In this study glutamine was the sole amino acid in the medium, while the present study was carried out in the presence of a complete broth of amino acids and the effect was obtained without heating. The manner by which glutamine leads to this effect is unknown, but underscores the possibility that some aspect of glutamine metabolism is involved in heat-shock protein expression.

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